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Characterization of luminescent *Vibrio campbellii* LZ5 and its potential application in the detection of environmental heavy metals

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Abstract.

A luminescent strain was isolated and identified as *Vibrio campbellii* strain LZ5 by 16S rDNA analysis. It grew well in broad living conditions, and the relative fluorescence intensity was stable at pH ranging from 5 to 7.5, NaCl concentrations from 0% to 3%, KCl from 1.5% to 5%, and $CaCl_2$ from 1% to 3%. In contrast, the relative fluorescence intensity was negatively correlated with both $CdCl_2$ and $HgCl_2$ concentrations from 0 to 1 mg/L. The luminescent fraction from the cell lysate was purified by ion-exchange chromatography, and identified as a luciferase by using matrix-assisted laser desorption/ionization–time of flight mass spectrometry. Furthermore, the biofilm of *V. campbellii* LZ5 was formed constantly under different conditions, and was not affected by heavy metal ions. The data collectively reveal that strain LZ5 has the potential to be developed into a biosensor for real-time monitoring of heavy metals.

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1. Introduction

Luminescent bacteria, living symbiotically with some deep sea organisms, emit light as a result of a chemoluminescence reaction catalyzed by luciferase in which chemical energy is converted into light energy. Currently, most of the isolated luminous strains belong to two genera of marine bacteria, *Vibrio* and *Photobacterium*. Because the expression levels of luciferase are influenced by growth, viability, and cellular metabolism in bacteria, the variation of emitted light, measured with a luminometer, can be used as an indicator for culture conditions. Indeed, a microbial assay based on the loss of bioluminescence from marine microorganisms, *Photobacterium phosphoreum* and *Vibrio fischeri*, has been developed into the commercialized Microtox system [1],[2]. Although Microtox assay (Strategic Diagnostics d/b/a SDIX, Newark, DE, USA) is rapid, reproducible, and sensitive to a broad range of inorganic and organic chemicals, the system has some shortcomings for applications in freshwater environments. For instance, it needs temperature control and preparation of culture media, which are laborious and inconvenient. Another example of a successfully developed biosensor, using the whole cells or purified luciferases, has been extensively utilized for monitoring organic pesticide and heavy metal contamination [3],[4]. One important attribute of a biosensor is the real-time process monitoring. Also, it is desirable to continuously supply a large amount of bacteria to the device. The results from previous research showed that luminescent intensity was affected by many factors, including oxygen and cell motility in a stirring batch culture [5], so it is hard to provide cells with constant properties, especially a stable luminosity. From this point of view, development of immobilized whole-cell preparations with a better storage and operational stability would be attractive. Some of the entrapment techniques for biosensors immobilize microbial cells on synthetic polymeric gels, including polyacrylamide [6], polyurethane-based hydrogels [7], and polyvinyl alcohol [8]. However, the complicated preparation process is costly and time consuming.

In the present study, a luminescent bacterium was isolated and identified as *Vibrio campbellii* strain LZ5 from the marine environment. It grew well under different pH and metal

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; OD, optical density; PBS, phosphate-buffered saline; $FMNH_2$, reduced flavin mononucleotide; CV, crystal violet; PAH, polycyclic aromatic hydrocarbon.

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conditions, and the relative fluorescence intensity remained stable. In contrast, the relative fluorescence intensity was negatively correlated with both $CdCl_2$ and $HgCl_2$ concentrations. The luminescent fraction was then purified on a diethylaminoethyl cellulose column (DEAE-52) (Beijing Propbs Biotechnology Co., Haidian, Beijing, China), and identified as a luciferase by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS). The significant biofilm formation of strain LZ5 was stable under different conditions. Characterization of strain LZ5 would be helpful for future development of biosensor monitoring of heavy metal ions in a toxicity measurement system.

2. Materials and methods

2.1. Isolation of luminescent bacterial strain and emission spectra analysis

Oysters (*Crassostrea gigas*) were purchased from a local supermarket. The oyster meat was separated and placed in a 0.9% NaCl (*m*/*v*) solution for 5 H at 30°C. Glowing colonies were selected and inoculated on a 2% agar plate containing 2216 E marine broth at the same temperature. The broth per liter was composed of NaCl (33.4 g), yeast extract (5 g), tryptone (10 g), KCl (1.5 g), $MgSO_4 \cdot 7H_2O$ (24.6 g), and $CaCl_2 \cdot 2H_2O$ (2.9 g).

Strain LZ5 conferred the best luminescence properties and was selected for further research. The spectral emission was recorded at 300–600 nm using a RF-5301PC spectrofluorophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) when strain LZ5 reached the exponential phase.

2.2. Identification of luminescent bacterial strain

Strain LZ5 was identified using a previously reported method [9]. The 16S rDNA gene was amplified with primers F27 5-AGAGTTTGATCATGGCTCAG-3 and R1492 5-TACGGTTACCTTGTTACGACTT-3. The PCR product was purified and ligated into pMD 19-T vector for further sequencing. The sequence of strain LZ5 was aligned using the EzTaxon program [10] available at http://www.eztaxon.org/.

2.3. Effects of pH and metal ions on bacterial growth

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solutions containing metal ions were sterilized by filtration through 0.22 μm Millipore filters (EMD Millipore Corporation, Billerica, MA, USA). The pH of 2216 E marine broth was adjusted with a MeterLab^{TM} PHM201 apparatus (Radiometer, Copenhagen, Denmark).

A luminescent strain was grown overnight in a 300 mL shake flask containing 25 mL of 2216 E marine broth at 30°C with shaking at 250 rpm. Fresh media (100 mL) with or without metal ions were inoculated with 2 mL of the overnight culture. The optical density (OD) of the cultures was then measured at 600 nm every time point using a 721 Visible Spectrophotometer (Wenzhou Xinfeng, Zhejiang, China). The bioluminescence was read by RF-5301PC spectrofluorophotometer (Shimadzu Scientific Instruments) set to excite at 350 nm and detected emission at 401 nm.

For the 96-well luminometer test of heavy metal ions, 190 μL/well of the culture was transferred to a 96-well plate into which 10 μL of the serially diluted heavy metal ion was added. Ten microliters of culture medium was used as control. The plate was incubated at 30°C, and then inserted into a 96-well luminometer (MLX; DYNEX Technologies, Chantilly, VA, USA) set at 30°C. The OD and bioluminescence were examined at each time point. The values were expressed as mean $\pm$ SEM ($P < 0.05$) of three independent observations for each time point.

2.4. Luciferase separation and MS analysis

Strain LZ5 was grown overnight in 2216 E marine broth in a 30°C air shaking incubator. The culture was diluted 100-fold using the same medium and grown until mid-log phase. The cells were harvested by centrifugation at 6,500*g* for 5 Min, and the pellet was washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and pH 7.5) and resuspended in an appropriate volume of cell lysis solution (25 mM Tris/HCl, 2 mM mercaptoacetic acid, 2 mM ethylenediaminetetraacetic acid, 1% triton X-100, and pH 7.2). The ultrasonic lysate was centrifuged, and 5 mL of supernatant was applied to a 1.7 $\times$ 100 cm^2 DEAE-52 column in PBS. The column was eluted with PBS at a rate of 0.5 mL/Min. The collected fractions were assessed for the luciferase activity by measuring the initial maximum light intensity after mixing with aldehyde, oxygen, and reduced flavin mononucleotide (FMNH2). The fractions with high luciferase activity were analyzed by SDS-PAGE, and the gel was stained with Coomassie Blue. The protein bands were excised from gel for MALDI–TOF MS analysis. In brief, the protein bands were digested with trypsin and mixed with matrix α-cyano-4-hydroxycinnamic acid (1:1), and then loaded onto a stainless steel MALDI plate. MS spectra were acquired using the ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Peptide mass maps were obtained in reflectron mode (1-keV accelerating voltage) with 1,000 laser shots per spectrum. Six external standards (mass standard kit for the 4700 Proteomics Analyzer calibration mixture, Part Number 4333604; Applied Biosystems) were included to calibrate each spectrum to a mass accuracy within 50 ppm. The resulting peptide masses were submitted to Mascot (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF) (Matrix Science, Boston, MA, USA) for NCBInr databases search.

2.5. Quantitative detection of crystal violet stained biofilm

The biofilm attached to the bottom of 96-well polystyrene plates was measured by spectrophotometry. In short, cells were grown overnight in Luria-Bertani medium, diluted to a final OD of 0.05 at $OD_{600\,nm}$, and then incubated in relevant medium described

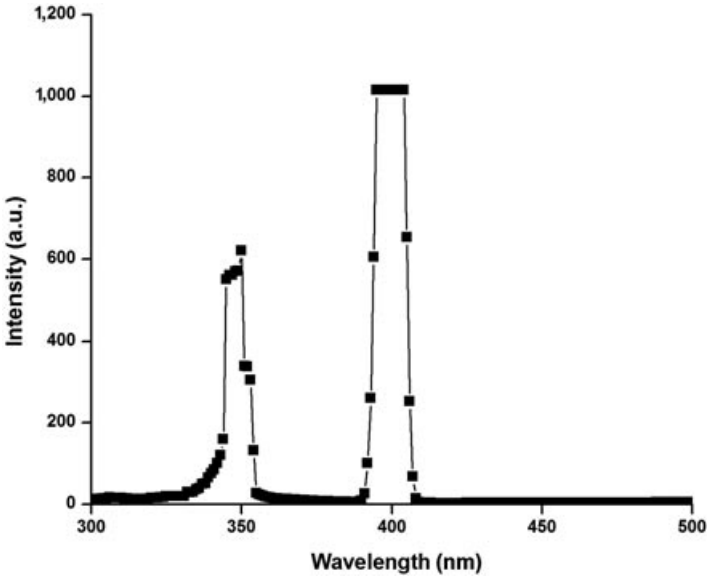

Fig. 1. Spectral emission of strain LZ5. The strain was grown in 2216 E marine broth at 30°C in batch conditions aerated by agitation at 300 rpm. The spectra were scanned when the A600 nm of cell culture was 0.3 by using a RF-5301PC spectrofluorophotometer.

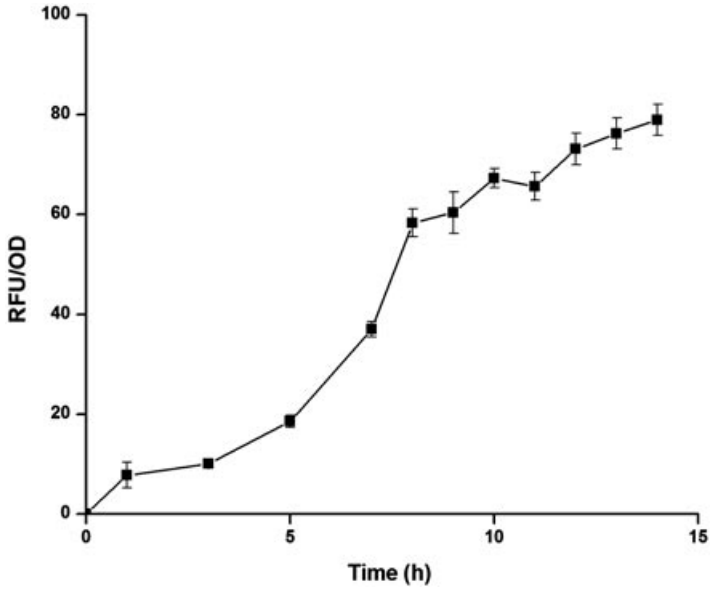

Fig. 2. Relative fluorescence intensity of strain LZ5 in different growth stages.

above in polystyrene 96-well plates (200 μL culture in each well) for 24 H without shaking. After treatment with different pH or metal ions, quantification of crystal violet (CV)-stained biofilms was carried out as follows: the supernatant was removed, and the wells were washed gently two times with isotonic phosphate buffer (0.15 M and pH 7.2). The adherent biofilm was stained with 0.1% CV (200 μL) and incubated at room temperature for 20 Min. The excess CV was cleansed by three gentle washes with distilled water. The remaining CV was solubilized with 95% ethanol, and the biofilm formation was quantified by measuring the absorbance at $OD_{570\ nm}$ with a microplate reader (BioTek, Winooski, VT, USA).

3. Results

3.1. Isolation and genetic identification of luminescent strain LZ5

The oyster meat was separated and placed in 0.9% NaCl (*m*/*v*) solution for 5 H at 30°C. Glowing colonies were selected and inoculated on a 2% agar plate containing 2216 E marine broth. One colony, which conferred the best luminescence properties, was named as strain LZ5. Spectral emission was recorded at 300–600 nm after incubating strain LZ5 until the exponential phase. Two peaks were clearly identified; the former was at 351 nm of the ultraviolet light range and the latter at 401 nm of the visible light (Fig. 1). Previous studies have shown that natural bioluminescent bacteria, for instance, *Vibrio harveyi* and *V. fischeri*, produced two peaks, one at 491–500 nm and the second at 585–595 nm, which could be related to the autofluorescence of luciferase [11]. Our results were different from the reported data, suggesting that strain LZ5 had specific luminescence spectra and was worthy of further study.

To genetically identify the genus or species, the 16S rDNA sequence of strain LZ5 was amplified by PCR, and the PCR products were purified and submitted directly for sequencing. The complete 16S rDNA gene sequence (1,475 bp) of strain LZ5 (GenBank accession number JN547725) was compared with the sequences from *V. campbellii* and other closely related taxa. The 16S rDNA gene sequence of strain LZ5 and *V. campbellii* American Type Culture Collection 25920(T) has a similar value of 99.862%. The results indicate that strain LZ5 is a member of *V. campbellii*.

3.2. Physiological conditions affecting growth and luminescence of strain LZ5

Strain LZ5 was grown in 2216 E marine broth at 30°C and monitored by hourly OD measurements. Before the measurement of fluorescence intensity, all samples were diluted to 0.05–0.10 OD to minimize any inner-filter effects. Fluorescence data were reported as relative fluorescence units per OD (RFU/OD) to evaluate luminescent expression on a per-cell mass basis. After 12 H incubation, the increasing rate of fluorescence intensity became constant (Fig. 2).

Next, the alteration of fluorescence intensity was assessed in different pH media after 12 H of incubation. As shown in Fig. 3a, strain LZ5 could grow in a wide pH range, and RFU/OD at pH ranging from 5 to 7.5 had no significant differences.

To evaluate the effects of metal ions, strain LZ5 was incubated in different concentrations of NaCl, KCl, or $CaCl_2$ for 12 H, and the light absorbance and the relative fluorescence were measured. The result showed that the growth and relative fluorescence intensity of strain LZ5 had no significant differences in 0%–3% NaCl concentrations (Fig. 3b). Similar results were

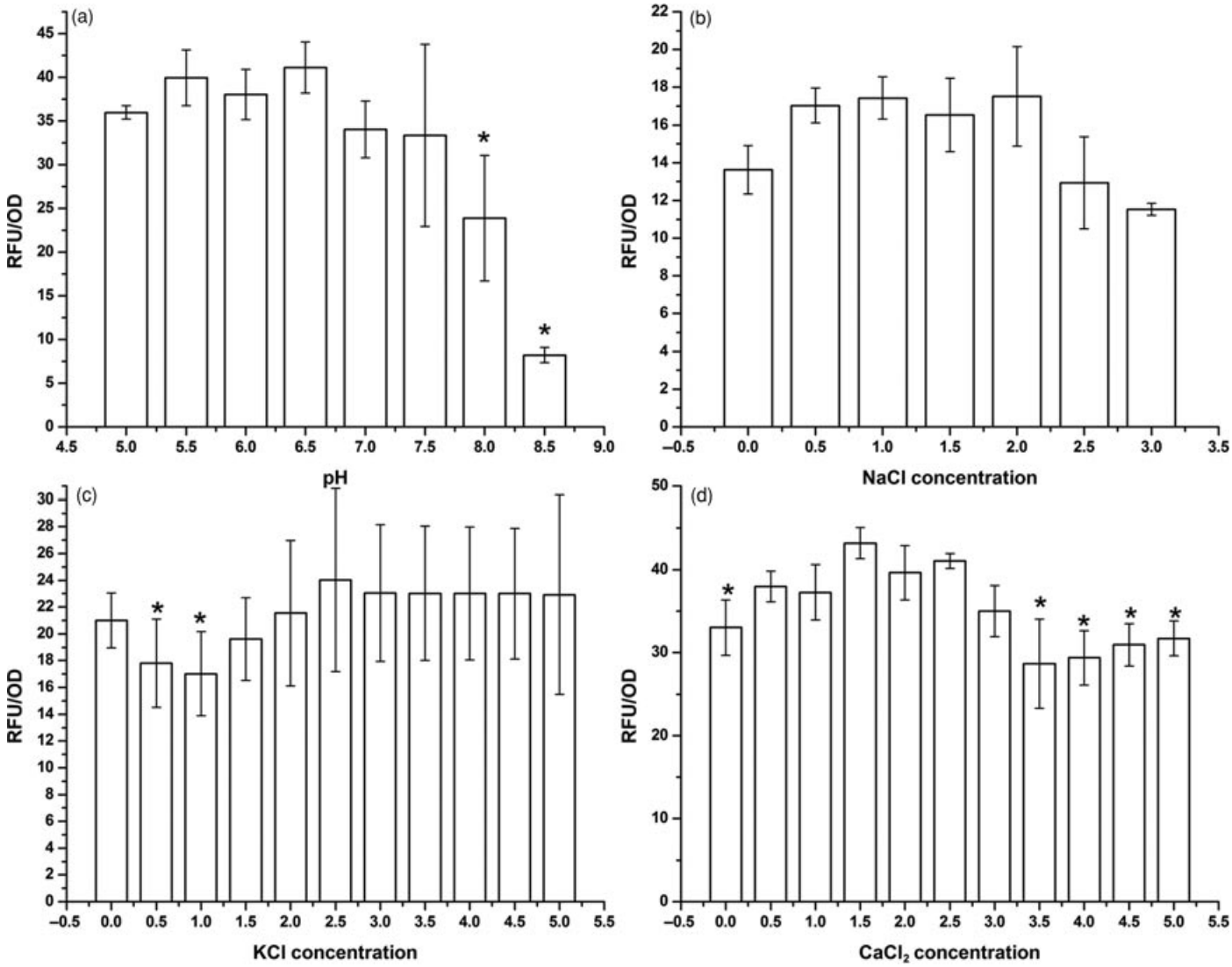

Fig. 3. Relative fluorescence intensity of strain LZ5 in different pH media (a), NaCl concentrations (b), KCl concentrations (c), and $CaCl_2$ concentrations (d). The values are presented as mean ± SEM of three independent observations. $P < 0.05$ was considered statistically significant according to the Scheffe test. $^*P < 0.05$ as compared with the values of other bars.

observed with different concentrations of KCl and $CaCl_2$ (Figs. 3c and 3d). The relative fluorescence intensity had no significant differences, with KCl ranging from 1.5% to 5%, or $CaCl_2$ ranging from 1% to 3%.

Finally, the effects of heavy metal ions were evaluated. For the $CdCl_2$ test, the cell cultures containing serially diluted heavy metal ions were assessed for OD and bioluminescence with a 96-well luminometer. After 75 Min of incubation, the relative fluorescence of strain LZ5 tended toward stable (data not shown); therefore, the bioluminescence in the presence of $CdCl_2$ was measured at 115 Min time point. As shown in Fig. 4a, the relative fluorescence reduced linearly with raising $CdCl_2$ concentrations. The $HgCl_2$ test was performed using the same method as described above. After 55 Min of incubation, the relative fluorescence reached the maximum (data not shown), and a negative linear relationship was observed between bioluminescence and $HgCl_2$ concentrations (Fig. 4b).

3.3. Luciferase separation and MS analysis

To investigate the underlying mechanism of bioluminescence, the lysate of strain LZ5 was collected and loaded on a DEAE-52 column for separation. Each fraction was used for luciferase assay. As shown in Fig. 5, tube 2 conferred the highest fluorescence intensity and therefore was chosen for SDS-PAGE analysis. Protein bands were extracted and identified as luciferase by MALDI–TOF MS (data not shown).

3.4. Effect of pH and heavy metal ions on biofilm formation of strain LZ5

To estimate the potential environmental application of *V. campbellii* LZ5, the biofilm development assays were carried out under different conditions. The biofilm production of strain LZ5 under different pH is shown in Fig. 6a. Biofilm formation of

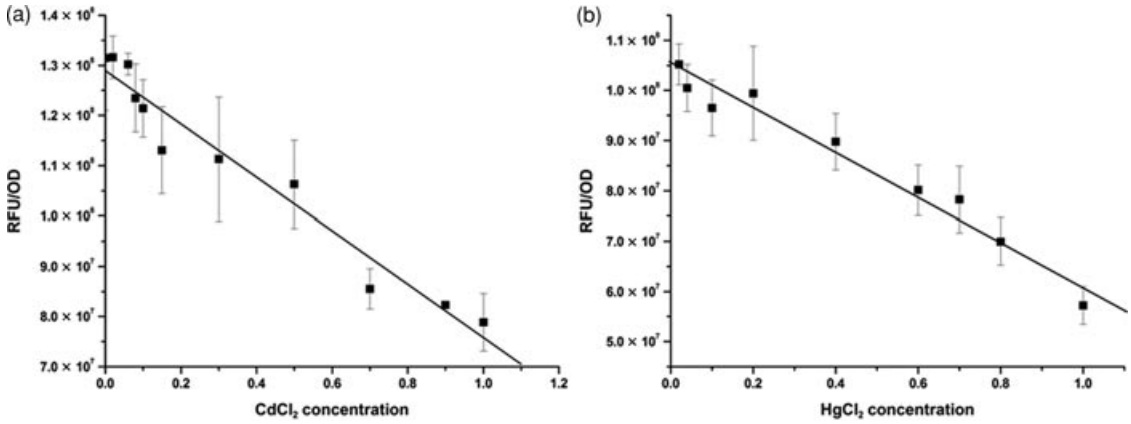

Fig. 4. Relative fluorescence of strain LZ5 in different $CdCl_2$ concentrations (a) and $HgCl_2$ concentrations (b).

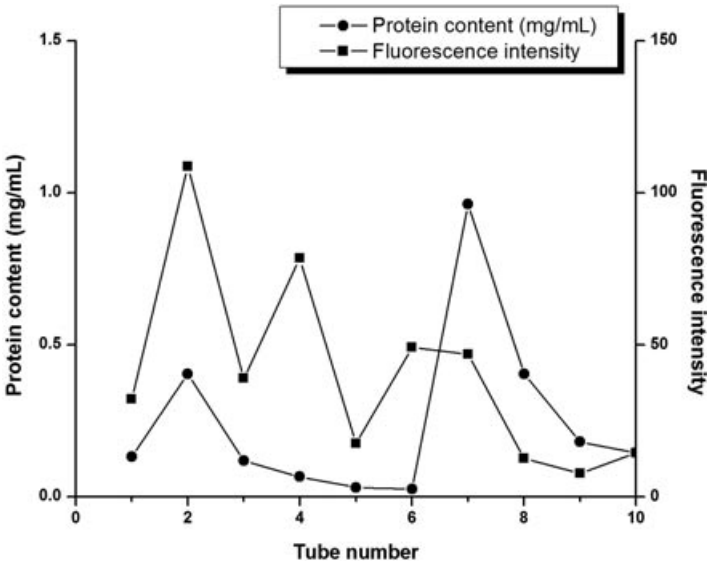

Fig. 5. Luciferase separation and fluorescence measurement.

strain LZ5 could be produced well in a wide pH range, and there were no significant differences at pH 5.5, 6.5, and 7.5.

To determine the effect of heavy metal ions on the biofilm production of LZ5, the 24 H biofilm of LZ5 was treated with $CdCl_2$ or $HgCl_2$ in concentrations ranging from 0 to 1.0 mg/L. After 2 H treatment, the biofilm formation was evaluated. As shown in Figs. 6b and 6c, the biofilm formation after treatment with different heavy ions was mostly unchanged and at fairly high levels. These results suggest that biofilm formation of LZ5 was very stable and not affected by heavy metal ions.

4. Discussion

In this study, a novel luminescent strain was isolated and identified as *V. campbellii* LZ5, which showed specific luminescent spectra that peaked at 351 and 401 nm. Because it was different from the reported luminescent strains *V. harveyi* and *V. fischeri*, which peak at 491–500 and 585–595 nm, respectively [12], we decided to characterize further the properties of the isolated strain. The growth and luminescence were observed in diverse physiological conditions.

The relative fluorescence of strain LZ5 was not significantly changed in the presence of NaCl, KCl, and $CaCl_2$. Next, the luminescence responses from strain LZ5 were studied with two different classes of pollutants, polycyclic aromatic hydrocarbons (PAHs), including naphthalene and benzo[a]pyrene, which cause toxicity and inhibit cellular metabolism; and heavy metals, including Hg or Cd, which are potential sources for metal bioaccumulation in cells. Interestingly, the relative fluorescence from cells treated with $CdCl_2$ or $HgCl_2$ was negatively correlated with ion concentrations. However, when cells were treated with PAHs, the relative fluorescence did not show a correlation with the organic chemical concentrations.

Previous studies have reported a whole-cell biosensor with a detection range from 50 to 1 mM for Cd [13] and an electrogenerated chemiluminescence biosensor for detection of Hg concentrations from 7.0 pM to 50 nM [14]. Strain LZ5 could measure up to 5.6 mM of Cd and 3.7 mM of Hg, which had a broader scope compared to those reported. The results therefore indicate that strain LZ5 is a good candidate for detection of heavy metals in the environment.

To investigate the mechanism of luminescence, the fluorescent fraction was purified and the reporter component was identified as a luciferase by MALDI–TOF MS analysis. All these data supported this characterization of strain LZ5 as helpful for future development of a biosensor for monitoring heavy metal ions in a toxicity measurement system. For this kind of equipment, bacteria must be provided continuously, which seems laborious. We were seeking a technique that could immobilize microbial cells on the synthetic polymers. Because the cells immobilized through adhesion were in direct contact with the liquid phase, mass transfer problems could therefore be eliminated. We found first that strain LZ5 could form a stable biofilm structure on polystyrene. The biofilm was then quantified with treatments of different pH and heavy ions. The outcomes displayed that the strain could form significant biofilm formation on 96-well polystyrene plates. The amount of biofilm was at a fairly high level and was not changed by

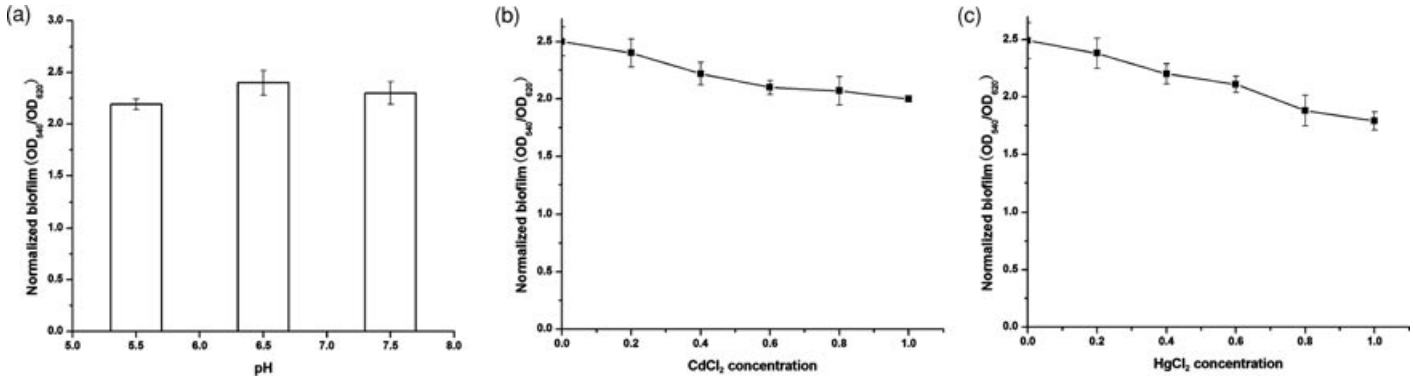

Fig. 6. Biofilm production of strain LZ5 in different pH media (a) and after treatment with different $CdCl_2$ concentrations (b) and $HgCl_2$ concentrations (c).

different pH. These results indicate that the biofilm formation of strain LZ5 was stable. In conclusion, the results in this study show that strain LZ5 is a promising candidate for development into a biosensor for the real-time process monitoring of heavy metals.

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